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Involvement of the telomeric protein Pin2/TRF1 in the regulation of the mitotic spindle

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Abstract Pin2/TRF1 was independently identified as a telomeric DNA-binding protein (TRF1) that regulates telomere length, and as a protein (Pin2) that can bind the mitotic kinase NIMA and suppress its lethal phenotype. We have previously demonstrated that Pin2/TRF1 levels are cell cycle-regulated and its overexpression induces mitotic arrest and then apoptosis. This Pin2/TRF1 activity can be potentiated by microtubule-disrupting agents, but suppressed by phosphorylation of Pin2/TRF1 by ATM; this negative regulation is critical in mediating for many, but not all, ATM-dependent phenotypes. Interestingly, Pin2/TRF1 specifically localizes to mitotic spindles in mitotic cells and affects the microtubule polymerization in vitro. These results suggest a role of Pin2/TRF1 in mitosis. However, nothing is known about whether Pin2/TRF1 affects the spindle function in mitotic progression. Here we characterized a new Pin2/TRF1-interacting protein, EB1, that was originally identified in our yeast two-hybrid screen. Pin2/TRF1 bound EB1 both in vitro and in vivo and they also co-localize at the mitotic spindle in cells. Furthermore, EB1 inhibits the ability of Pin2/TRF1 to promote microtubule polymerization in vitro. Given that EB1 is a microtubule plus end-binding protein, these results further confirm a specific interaction between Pin2/TRF1 and the mitotic spindle. More importantly, we have shown that inhibition of Pin2/TRF1 in ataxia–telangiectasia cells is able to fully restore their mitotic spindle defect in response to microtubule disruption, demonstrating for the first time a functional involvement of Pin2/TRF1 in mitotic spindle regulation.

Key words: ATM; Pin2/TRF1; Microtubule; Mitotic spindle checkpoint; Telomere

1. Introduction

Telomeres are essential for preserving chromosome integrity during cell division. Telomeres are composed of repetitive DNA sequences of TTAGGG arrays concealed by a complex of telomeric proteins that protects the ends from exonucleolytic attack, fusion and incomplete replication [1–5]. An increasing body of evidence links telomeres to mitotic progression. For example, deletion of telomeres triggers mitotic arrest and apoptosis in *Drosophila* [6]. In fission yeast, telomeres are clustered at the nuclear periphery in G2, but this association is disrupted in mitosis [7], and telomeres have been shown to mediate the attachment of chromosomes to spindle bodies and lead chromosome movement in meiotic prophase [8]. In budding yeast, mutations in the related *TEL1* and MEC1 genes result in shortened telomeres, G2/M checkpoint defect and genomic instability [9,10]. Similarly, mutations in its human counterpart, the ATM gene, cause ataxia–telangiectasia (A-T) both in humans and mice, displaying a wide range of abnormalities, including those related to telomere dysfunction [11–15]. More interestingly, cell lines derived from A-T patients have shortened telomere lengths [16–18] and defective mitotic checkpoints [19,20]. Finally, mutations in the *Tetrahymena* telomeric DNA sequence have been shown to cause a block in anaphase chromosome separation [21]. Collectively, these results suggest that telomeres may be important for regulation of mitosis. However, little is known about the identity and function of the signaling molecule(s) involved in this process.

In our effort to understand molecular mechanisms of mitotic regulation, we previously isolated three human proteins, Pin1–3, that physically and functionally interact with NIMA, an essential mitotic kinase in *Aspergillus nidulans* [22,23]. Characterization of these Pin proteins shows that they are all involved in mitotic regulation [24]. Pin1 binds and regulates the function of a subset of phosphoproteins by controlling the conformation of specific phosphorylated Ser/Thr–Pro motifs [25–29]. Pin2 is identical in the sequence to TRF1 apart from an internal deletion of 20 amino acids [30]. TRF1 is a double strand DNA-binding protein that negatively regulates telomere elongation [31,32]. Pin2 and TRF1 are likely to be two alternatively spliced isoforms of the same gene *PIN2/TRF1*, as suggested by Young et al. [33]. However, we have shown that Pin2 is 5–10-fold more abundant than TRF1 in the cells and the expression level of Pin2/TRF1 is tightly regulated during the cell cycle [30]. Both Pin2 and TRF1 contain a D-like motif similar to the destruction box present in many mitotic proteins, and their protein levels are significantly increased in late G2 and mitosis and then degraded as cells exit from mitosis [30]. Furthermore, overexpression of Pin2/TRF1 induces abortive mitosis and apoptosis in cells containing short telomeres [34]. Moreover, Pin2/TRF1 is an ATM kinase substrate and inhibition of Pin2/TRF1 by ATM plays a critical role in ATM-dependent regulation of telomere maintenance, the radiosensitivity and G2/M checkpoint regulation [35,36]. Interestingly, the localization of Pin2/TRF1 is regulated during the cell cycle; Pin2/TRF1 specifically localizes to mitotic spindles in mitotic cells [37]. These results indicate that

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Pin2/TRF1 has a specific function in mitotic regulation. However, little is known about how Pin2/TRF1 is involved in mitotic progression and whether Pin2/TRF1 affects the microtubule function in mitotic cells.

As part of our effort to further elucidate how Pin2/TRF1 is involved in regulation of telomeres and mitotic regulation, we have recently employed a yeast two-hybrid screen and identified six known genes and four unknown PinX1–4 genes, whose products interact with this protein [38]. Characterization of PinX1 reveals that it is a potent catalytic inhibitor of telomerase and a putative tumor suppressor [38]. These results further support a negative role of Pin2/TRF1 in telomere regulation and the role of telomerase in oncogenesis.

One known gene identified in our Pin2/TRF1 two-hybrid screen is EB1. Human EB1 interacts with the tumor suppressor APC in a cell cycle-dependent manner [39–41]. This interaction targets APC to the microtubule distal tips [42,43] and is critical for promoting microtubule assembly [44]. The EB1 homologues Mal3 and BIM1 in both budding and fission yeast, respectively, have also been shown to promote microtubule assembly [41,45,46]. Finally, Bim1p is involved in regulation of the late mitotic checkpoint [47]. Mutation of BIM1 abolishes the delay following improper separation of chromosomes, resulting in multinucleated cells. Because aneuploidy is often observed in colon cancer cells [48], the late mitotic checkpoint has been proposed to be responsible for the tumor suppressive effect of APC [49].

Here we characterized interaction of Pin2/TRF1 and EB1 and examined the functional significance of Pin2/TRF1 in mitotic spindle regulation in vivo. Pin2/TRF1 bound EB1 both in vitro and in vivo and they also co-localize at the mitotic spindle in cells. Furthermore, EB1 inhibits the ability of Pin2/TRF1 to promote microtubule polymerization in vitro. Moreover, we have shown that inhibition of Pin2/TRF1 in A-T cells is able to fully restore their mitotic spindle defect in response to microtubule disruption. These results confirm a specific interaction between Pin2/TRF1 and the mitotic spindle, and more importantly demonstrate for the first time a functional involvement of Pin2/TRF1 in mitotic spindle regulation.

2. Materials and methods

2.1. DNA construction

Various GFP-Pin2 constructs were described previously [34]. To construct GFP-Pin2$^{300-419}$, GFP-Pin2 plasmid was digested by *Eco*RI and *Xmn*I, followed by self-ligation. Various GST fusion proteins were expressed in bacteria and purified, as described [29].

2.2. Fluorescence microscopy

Fluorescence microscopy was performed, as described previously [37,44]. Briefly, HeLa cells were transfected by pEGFP and RFP-EB1 constructs and fixed with methanol, subjected to fluorescence microscopy.

2.3. Microtubule polymerization assays

Microtubule assembly assay was performed, as described [28,37,44]. Briefly, 0.77 μM of various recombinant proteins was incubated with 13 μM of purified bovine tubulin (cytoskeleton) in 20 μl of microtubule stabilizing buffer containing 1 mM GTP, 1 mM $MgCl_2$ and 10% glycerol at 30°C, followed by measuring changes in turbidity every 6 s.

2.4. Stable expression of ATM or Pin2 mutants in A-T cells

For stable expression of ATM, pEBS7 vector encoding full length ATM tagged with FLAG or the control vector were stably transfected into parental A-T22IJE-T cells, as described [50]. A-T22IJE-T cells were originally derived from primary ACT fibroblasts, which harbor a homozygous frameshift mutation at codon 762 of the ATM gene and contain no ATM protein since the truncated protein is not stable [50–52]. After selection with hygromycin B (200 μg/ml) and limiting dilution, multiple clones were isolated and checked for ATM expression by immunoblotting analysis with anti-ATM antibody (Ab-3) and anti-FLAG antibody (M5). For stable expression of the Pin2 mutant, cDNA encoding Pin2$^{1-316}$ was cloned into the pEGFP-C1 vector and stably transfected into A-T22IJE-T cells. After selection with G418 (1 mg/ml), GFP-expressed cells were picked up under fluorescence microscopy. Multiple stable clones were obtained with similar properties.

2.5. Cell cycle analysis

To examine the effects on the mitotic spindle checkpoint, cells were incubated with 100 ng/ml Nocodazole for 16–20 h and harvested, fixed and stained by propidium iodide (0.01 mg/ml) containing 0.25 mg/ml of RNase, followed by flow cytometrical analysis (Becton-Dickinson), as described [34,35].

2.6. Histone H1 kinase assay

Histone kinase assay was performed as described previously [22]. Briefly, cell extracts were incubated with 200 μM [γ-^{32}P]ATP and 2.5 μg histone H1 at 23°C for 10 min and the reaction was stopped by addition of SDS sample buffer, followed by SDS–PAGE and autoradiography.

3. Results and discussion

3.1. Pin2/TRF1 interacts with EB1 in vitro and in vivo

In our search for Pin2/TRF1-binding proteins by two-hybrid screening of a HeLa cDNA library [38], we identified three independent clones encoding EB1. GST pulldown was used to confirm their direct interaction in vitro. [^{35}S]Pin2 was generated by in vitro transcription and translation, followed by GST pulldown assay with purified GST-EB1. Although there was no binding between GST and Pin2, GST-EB1 readily bound Pin2 (Fig. 1a). To examine whether EB1 binds cellular Pin2/TRF1, lysate of HCT116 human colon cancer cells containing wild-type APC was subjected to the GST-EB1 pulldown assay. As expected, APC was precipitated by EB1 (Fig. 1b). More importantly, endogenous Pin2/TRF1 was also precipitated by EB1, but not by GST (Fig. 1b). Finally, to examine whether EB1 and Pin2 form a stable complex in cells, HA-Pin2/TRF1 and FLAG-EB1 constructs were co-transfected into HeLa cells. Then their lysate was subjected to immunoprecipitation with anti-FLAG monoclonal antibody (mAb), followed by immunoblotting with anti-FLAG (EB1) and anti-HA (Pin2/TRF1) mAbs. As shown in Fig. 1c, Pin2 was co-precipitated with EB1. These results demonstrate that EB1 interacts with Pin2/TRF1 both in vivo and in vitro.

3.2. Pin2/TRF1 co-localizes with EB1 at the mitotic spindle

To further confirm the in vivo interaction between Pin2/TRF1 and EB1, we co-transfected GFP-Pin2 and RFP-EB1 constructs into HeLa cells and examined their localization during the cell cycle using fluorescence microscopy. As shown previously [37], Pin2/TRF1 binds microtubules and localized to the mitotic spindle only in mitotic cells (Fig. 2). Interestingly, GFP-Pin2 and RFP-EB1 co-localized in mitotic spindles only in these mitotic cells (Fig. 2), but not in interphase cells (data not shown). These results indicate that Pin2/TRF1 and EB1 not only bind, but also co-localize at the mitotic spindle in the cell.

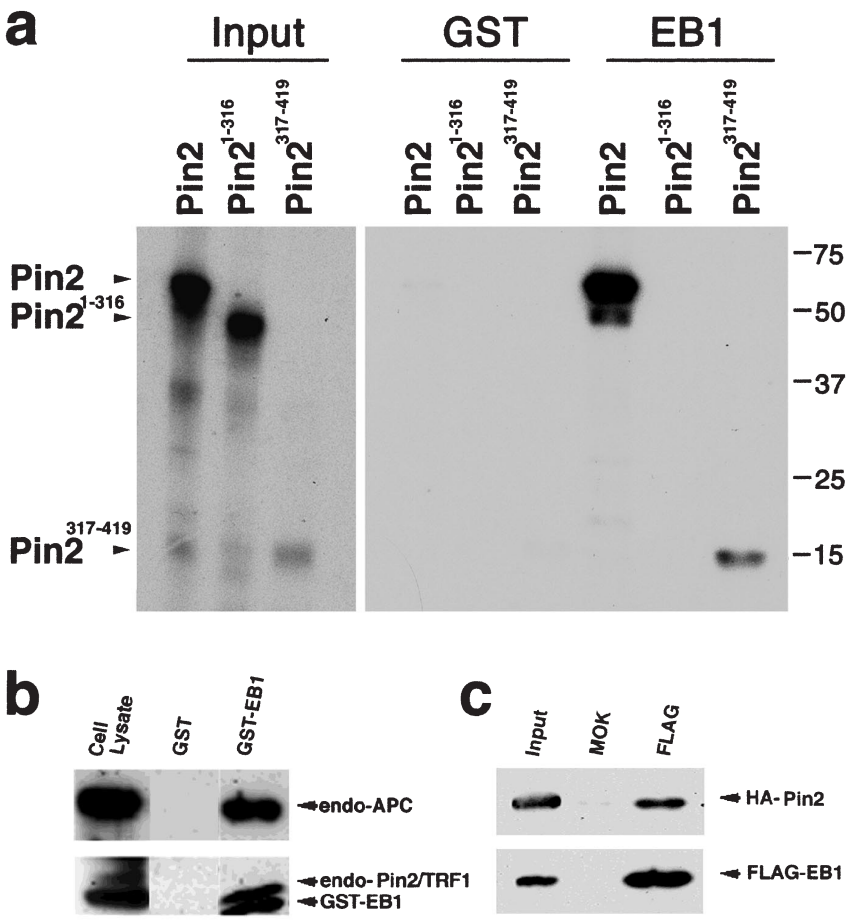

Fig. 1. EB1 binds Pin2/TRF1 in vitro and in vivo. a: GST-EB1 pulldown with translated [^{35}S]Pin2/TRF1. [^{35}S]Pin2/TRF1 was produced and incubated with interphase or mitotic *Xenopus* extracts followed by incubation with glutathione beads containing GST or GST-EB1. After wash, bound Pin2/TRF1 was separated on SDS gels, followed by autoradiography. b: GST-EB1 pulldown with HCT116 cell lysate. HCT116 colon cancer cells were used as the source of endogenous APC and Pin2/TRF1 proteins. Cells were lysed, and incubated with beads containing GST or GST-EB1. Endogenous APC and Pin2/TRF1 (endo-APC and endo-Pin2/TRF1) were detected by Western blotting. GST-EB1 made a band just below Pin2/TRF1 as a background of Western blotting. c: Co-immunoprecipitation of EB1 and Pin2/TRF1 in vivo. HeLa cells were co-transfected with constructs expressing HA-Pin2/TRF1 and FLAG-EB1, then subjected to immunoprecipitation with anti-FLAG mAb, followed by immunoblotting analysis with anti-FLAG or anti-HA mAb.

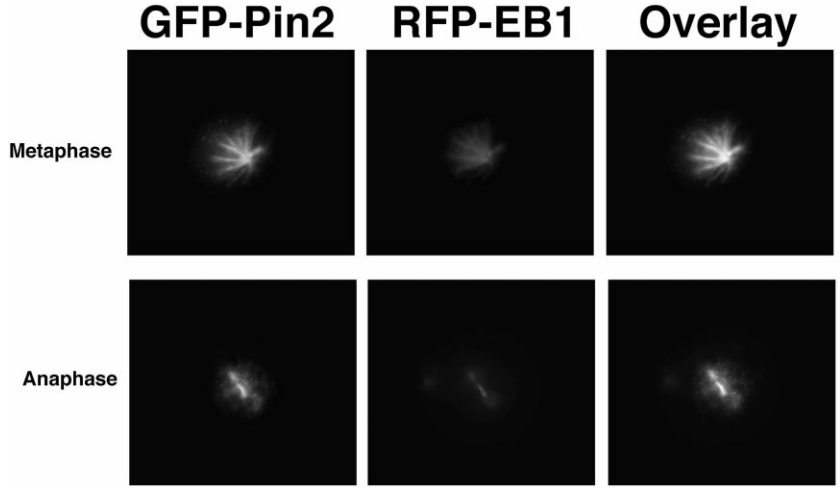

Fig. 2. EB1 and Pin2 co-localize in mitotic cells in vivo. GFP-Pin2/TRF1 and RFP-EB1 were co-transfected into HeLa cells. Cells were harvested before apoptosis and subjected to fluorescence microscope.

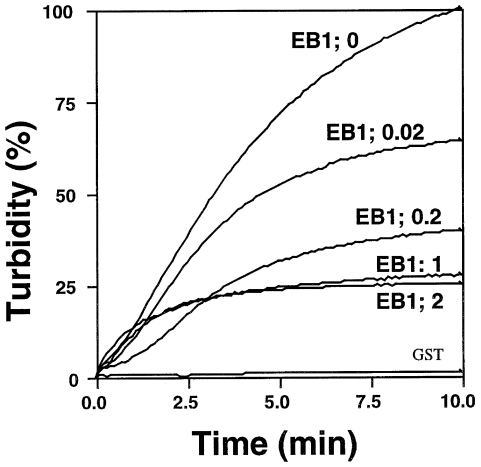

Fig. 3. EB1 suppresses the ability of Pin2 to promote microtubule polymerization in vitro. Pin2 was incubated with purified tubulin and increasing concentrations of EB1, and the microtubule assembly was measured by changes in the turbidity. The turbidity in the presence of Pin2 alone at 10 min is defined as 100%. Indicated concentrations of EB1 are relative molar ratio with Pin2.

3.3. *EB1 inhibits the ability of Pin2/TRF1 to promote microtubule polymerization in vitro*

We have previously demonstrated that Pin2/TRF1 binds microtubules and promotes microtubule polymerization in vitro [37]. An obvious question is that EB1 has any effect on microtubule polymerization activity of Pin2/TRF1. To address this question, we performed in vitro microtubule assembly with Pin2 and EB1. Pin2 and EB1 proteins were incubated with purified tubulins in microtubule stabilizing buffer. The assembly of microtubules was measured by changes in the turbidity, as described previously [37,44]. Pin2 alone promoted microtubule polymerization (Fig. 3), as previously reported [37]. Interestingly, EB1 suppressed this ability of Pin2 (Fig. 3). Furthermore, this inhibitory effect depended on the molar ratio of EB1 with Pin2 (Fig. 3). These results indicate that EB1 can inhibit the ability of Pin2 to affect microtubule polymerization in vitro.

3.4. *Inhibition of Pin2/TRF1 in ATM-negative cells restores their defect in response to disruption of the mitotic spindle*

Given the in vitro and in vivo interaction between Pin2/TRF1 and EB1, it is important to examine whether Pin2/TRF1 affects any microtubule-related function during mitosis in vivo. A-T (ATM-negative) cells display several cell cycle defects, including a prominent mitotic defect in response to microtubule disruption [53–57]. As shown in Fig. 4a, A-T cells, A-T22IJE-T [50,58], failed to arrest at mitosis in response to the microtubule-disrupting agent Nocodazole. Instead, they entered apoptosis. Importantly, this phenotype was fully rescued by stable re-expression of ATM (Fig. 4a), indicating that the defect is specifically due to loss of ATM. We have previously demonstrated that Pin2/TRF1 is an ATM substrate that plays a critical role in mediating ATM regulation [35,36]. Inhibition of Pin2/TRF1 function complements telomere shortening, the radiosensitivity and G2/M checkpoint defect of A-T cells [36]. Moreover, overexpression of Pin2/TRF1 induces mitotic entry and apoptosis, and this activity is significantly potentiated by Nocodazole treatment or by ATM mutations [34,35]. These results suggest that Pin2/TRF1 might be involved in the mitotic spindle defect in A-T cells.

To address this question, we inhibited the function of endogenous Pin2/TRF1 in A-T cells by stable expression of a dominant-negative Pin2/TRF1 mutant, GFP-Pin2$^{1-316}$, with the vector GFP as a control, as described previously [36]. To examine the effect of the mitotic spindle disruption, cells were treated with Nocodazole or Taxol or control buffer for 16 h and the cell cycle profiles were determined by flow cytometry. In the absence of Nocodazole or Taxol, there was no detectable difference in the cell cycle profile between GFP-Pin2$^{1-316}$-expressing and vector-transfected cells (Fig. 4b). However, after Nocodazole treatment, like vector control A-T cells (Fig. 4a), only about 50% of GFP control cells were accumulated with the 4n DNA content and ~23% of cells contained the sub-G1 DNA content indicative of apoptosis (Fig. 4b). In sharp contrast, almost all cells expressing GFP-Pin2$^{1-316}$ were accumulated with the 4n DNA content (Fig. 4b), as A-T cells that re-expressed ATM (Fig. 4a). Similar results were also obtained with Taxol (data not shown). These results suggest that expression of Pin2$^{1-316}$ in A-T cells may restore their mitotic spindle defect.

Cells with the mitotic defects have been shown to exit mitosis prematurely without cytokinesis after treatment with microtubule-disrupting agents; these cells contain the 4n DNA content with decondensed interphase nuclei or eventually enter apoptosis [59–61]. To further confirm that Pin2$^{1-316}$ indeed restores the mitotic defect in response to microtubule disruption in A-T cells, we observed the nuclear morphology using DAPI staining and assayed Cdc2 kinase activity using histone H1 as a substrate. After Nocodazole treatment, GFP control cells contained decondensed interphase nuclei, with about 20% cells containing apoptotic nuclei (Fig. 4c), consistent with the results obtained from flow cytometrical analysis (Fig. 4b). Furthermore, the Cdc2 kinase activity was much lower than that present in mitotic HeLa cells (Fig. 4d). These results are consistent with the fact that these A-T cells lack the mitotic spindle checkpoint; they exit mitosis prematurely without cytokinesis or enter apoptosis after Nocodazole treatment. In contrast, most GFP-Pin2$^{1-316}$-expressing cells contained condensed chromosomes and elevated Cdc2 kinase activity similar to that present in mitotic HeLa cells (Fig. 4c,d), both of which are characteristic of a sustained mitotic block. These results together suggest that stable expression of the Pin2 mutant in A-T cells is able to restore their mitotic spindle defect, demonstrating the functional significance of the interaction between Pin2/TRF1 and the mitotic spindle.

Our results demonstrate the physical and functional interaction between Pin2/TRF1 and the microtubule-binding protein EB1, and also suggest a novel role of Pin2/TRF1 in the regulation of the mitotic spindle. This is consistent with previous studies linking telomeres to mitotic progression in other model systems. For example, mutations or deletion of the telomeric DNA sequence triggers mitotic entry and apoptosis in *Drosophila* [6] or causes a severe delay or block in anaphase in *Tetrahymena* [21]. Furthermore, it is also supported by the cell cycle-specific regulation of Pin2/TRF1 function [30,34,35], and by the microtubule-binding activity of Pin2/TRF1 [37]. At present, the significance of binding of EB1 and Pin2/TRF1 is unclear, and we can only speculate how expression of the mutant Pin2$^{1-316}$ is able to restore the mitotic spindle checkpoint defect in A-T cells and how Pin2/TRF1 is normally

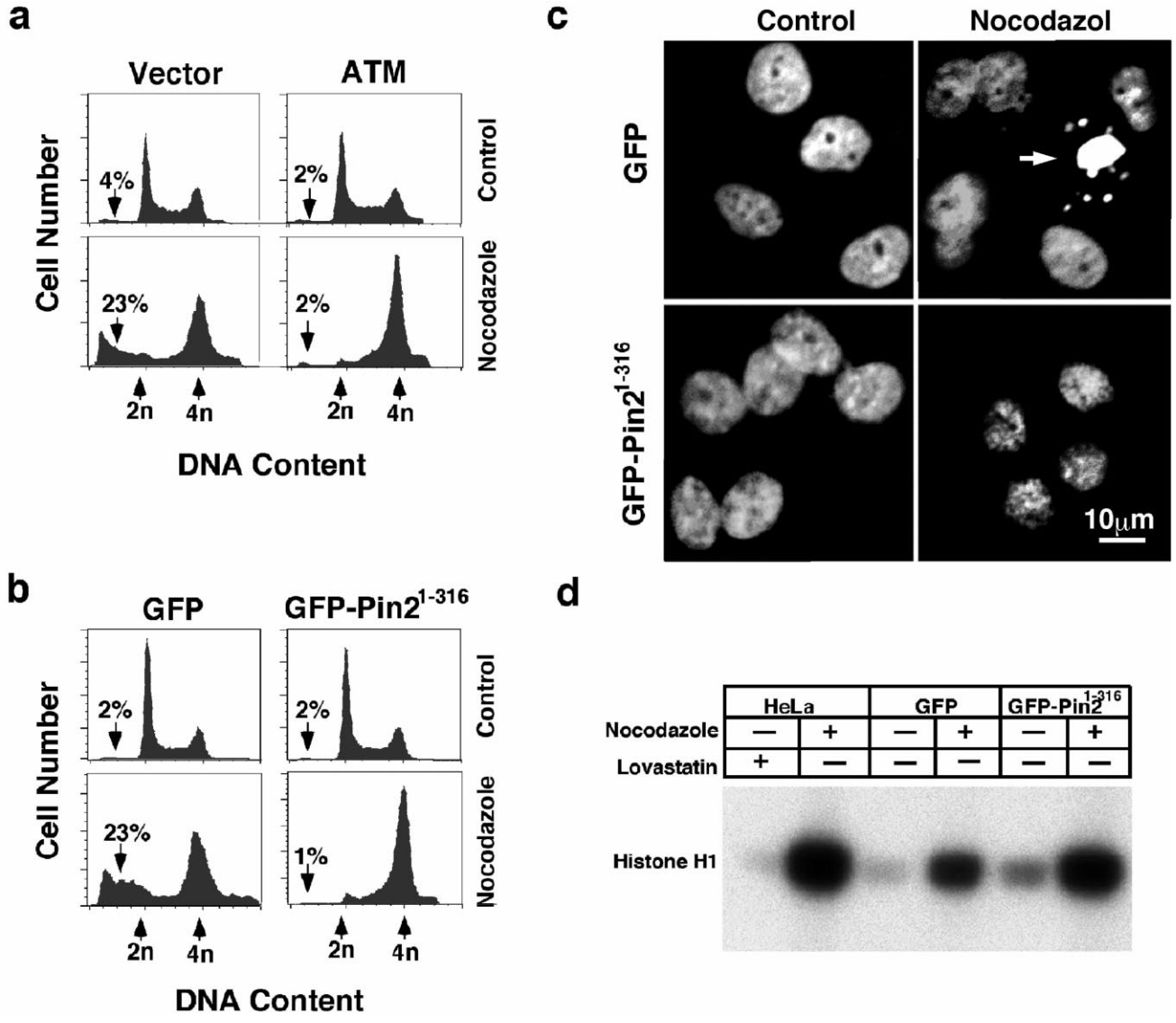

Fig. 4. Inhibition of Pin2/TRF1 rescues the spindle checkpoint defect in A-T cells. a: ATM rescues the spindle checkpoint defect in A-T cells. A-T cells stably expressing ATM or the control vector were incubated for 16 h in the presence of Nocodazole or control buffer (control), followed by flow cytometry. b: GFP-Pin2$^{1-316}$ but not GFP rescues the normal response to Nocodazle in A-T cells. Stable A-T cell lines were incubated with Nocodazole or control buffer (control) for 16 h and then subjected to flow cytometry. c: GFP- but not GFP-Pin2$^{1-316}$-expressing cells exit mitosis or enter apoptosis after Nocodazole treatment. Stable A-T cell lines were incubated for 16 h in the presence of Nocodazole or control buffer and then stained with DAPI. Arrow points to an apoptotic cell. d: GFP-Pin2$^{1-316}$- but not GFP-expressing cells contain the mitotic level of Cdc2 activity after Nocodazole treatment. Stable A-T cell lines were treated with Nocodazole (+) or control buffer (−) for 16 h and cell extracts prepared, followed by assaying Cdc2 kinase activity using histone H1 as a substrate. HeLa cells that were arrested in G1/S or M by Lovastatin or Nocodazole, respectively, were used as controls.

involved in the mitotic spindle checkpoint. Since Pin2$^{1-316}$ does not bind microtubules or promote microtubule polymerization [37], its effect on the mitotic spindle checkpoint is likely via inhibiting endogenous Pin2/TRF1 function in a dominant-negative manner, as shown previously [32,36]. This is also supported by our findings that expression of Pin2$^{1-316}$ significantly reduces the microtubule-binding activity of endogenous Pin2/TRF1 in A-T cells [37]. At entry into mitosis, the breakdown of the nuclear envelope and the increase in Pin2/TRF1 levels, which normally occurs at this time of the cell cycle, allow Pin2/TRF1 to bind microtubules. This interaction targets Pin2/TRF1 to the mitotic spindle, where it may affect the function of microtubules or other proteins on the mitotic spindle and thereby involve mitotic checkpoint regulation. Further experiments are needed to elucidate how Pin2/TRF1 is involved in the mitotic spindle checkpoint.

In summary, we have shown that Pin2/TRF1 interacts with the microtubule plus end-binding protein EB1 both in vitro and in vivo. Furthermore, EB1 inhibits the ability of Pin2/TRF1 to promote microtubule polymerization in vitro. Moreover, inhibition of Pin2/TRF1 mutant in A-T cells is able to fully restore their mitotic spindle defect in response to microtubule disruption. These results not only confirm the specific interaction between Pin2/TRF1 and the mitotic spindle, as we demonstrated earlier, but also demonstrate for the first time a functional importance of Pin2/TRF1 in the regulation of the mitotic spindle.

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